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(57) Abstract

The present invention relates to a membrane bound form of ST2 protein known as ST2L protein, particularly the development of reagents such as antibodies, ligands, agonists and/or antagonists specific for ST2L which may be used in cell separation or identification, drug screening and treatment of ailments such as infections, inflammation, and allergy.

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REAGENTS SPECIFIC FOR ST2L AND USES THEREFOR

The present invention relates to a membrane bound form of ST2 protein known as ST2L protein, particularly the development of reagents specific for ST2L which may be used in cell separation or identification, drug screening and treatment of ailments such as infections, inflammation, and allergy.

$CD4^+$ T cells can be divided into Th1 and Th2 subsets, the balance of which frequently determines the outcome of infections and autoimmune diseases. Th1 cells characteristically produce interferon gamma (IFN-γ) and activate macrophages to kill intracellular pathogens and can cause inflammatory diseases. Th2 cells produce interleukin (IL)-4 and IL-5 and are associated with allergic reactions involving IgE, eosinophils and basophils [reviewed in Mosmann, T.R. & Coffman, R.L. (1989) Ann. Rev. Immunol. 7, 145-173]. However, a stable cell surface marker distinguishing Th1 cells from Th2 cells has hitherto not been found and the mechanism for selective cytokine induction is not known.

Cloning of a cDNA encoding murine ST2 protein has previously been reported by Tominaga (1989) [FEBS Letters 258, 201-304]. The ST2 protein was reported to be a member of the immunoglobulin superfamily and to be highly similar to the extracellular portion of mouse interleukin 1 receptor.

Takagi et al (1993) [Biochimica et Biophysica Acta 1178, p194-200] identified a product of the murine ST2 gene in growth-stimulated BALB/C-3T3 cells and NIH 3T3 cells. The murine ST2 cDNA was transiently expressed in COS7 cells and the ST2 protein secreted in the culture fluid. The ST2 protein was also detected in the medium of BALB/C-3T3 cells and NIH 3T3 cells.

Yanagisawa et al (1993) [FEBS Letters 258, 83-87] describes a cDNA encoding a membrane-bound form of the mouse ST2 protein, which was designated "ST2L". In addition to the signal peptide and extracellular domains of the ST2 protein, ST2L comprises additional 3' sequence encoding a transmembrane region and a cytoplasmic region. The amino acid sequence of the extracellular domain of ST2L was found to be 25% identical to the extracellular domain of murine type I IL-1.

Yoshida et al (1995) [Hybridoma 14 p419-428], discloses eight species of murine monoclonal antibodies against human ST2 protein. All the antibodies reacted with N-glycosylated ST2 protein secreted from COS7 cells. The antibodies were found to react with a membrane bound chimeric molecule possessing an extracellular portion of human ST2. The human leukemic cell line UT-7 was also shown to express ST2 protein.

The present invention is based in part on the discovery by the present inventors that the ST2L protein is expressed on the surface of certain classes of T cells but not on other T cells.

The present inventors have utilised a technique known as differential display-polymerase chain reaction (DD-PCR), see Liang, P. and Pardee, A.B. (1992) Science 257, 967-971, to identify a gene encoding a cell membrane bound molecule, originally designated ST2L (see Yanagisawa, K., et al (1993) FEBS 318, 83-87) and expressed constitutively and stably on murine Th2 cells but not on Th1 cells. Yanagisawa et al (1997) [J. Biochem 121 p95-103] discloses that murine ST2 and ST2L mRNA are preferentially expressed by Th2 and D10 cells, but not by Th1 cells. Studies using the human ST2 protein showed that ST2L was believed to be expressed on the surface of at least one fraction of lymphocytes in peripheral blood mononuclear cells. However, Yanagisawa et al did not attempt to define which sub-population of lymphocytes expressed ST2L on their surface.

The present inventors have also shown a membrane bound form of human ST2 (hereinafter referred to as ST2L in line with the murine protein) to be expressed constitutively and stably on human Th2 cells but not on Th1 cells. Furthermore, the inventors have identified the expression of ST2L on type 2 cytotoxic T cells (Tc2) but not Tc1 cells. Thus, ST2L is expressed on type 2 T cells which typically produce IL-4 and IL-5 and not on type 1 T cells which produce IFN-γ. However, for the purpose of illustration the present description will refer principally to Th2 and Th1 cells, but it should be appreciated that the description is not limited thereto and may be similarly equated with Tc2 and Tc1 cells also.

In a first aspect, the present invention provides a method for distinguishing cells which express membrane bound ST2L from those that do not, the method comprising contacting a sample of cells with a reagent specific for ST2L and determining the presence or absence of an ST2L/reagent complex.

It will be appreciated that membrane bound ST2L possesses intracellular and extracellular domains as well as a transmembrane region and that the reagent specific for ST2L is generally specific for the extracellular domain.

Typically Th2/Th1 cells may be distinguished using the above method. Since ST2L is present on Th2 and not Th1 cells, the above method distinguishes Th2 cells by the presence of an ST2L/reagent complex and Th1 cells by the absence of an ST2L/reagent complex. Thus, such a method may also be used to identify whether or not a sample of activated Th cells are of the Th2 or Th1 type. Such a method represents a simple means of distinguishing between Th1 and Th2 cells and/or detecting the presence of Th2 cells in a sample.

In an embodiment, the present invention also provides a method for purifying Th1 or Th2 cells from a sample comprising Th1 and/or Th2 cells, the method comprising contacting a sample comprising Th1 and/or Th2 cells with reagent specific for ST2L, such that any Th2 cells present in the sample bind to the reagent, and removing any unbound material.

In this manner Th1 cells present in the sample will remain with the unbound material and thereby be purified away from Th2 cells. Subsequently Th2 cells may be separated from the ST2L specific reagent by washing, eluting or other conventional means of removal, thereby providing a purified sample of Th2 cells. Such a method may also be extended to a sample of cells comprising activated Th cells (ie. Th1 or Th2) and used to identify such cells and allow their purification.

Typically the reagent specific for ST2L may be bound to a substrate such as a plastics substrate, microcarrier beads or a chromatography support media.

Another aspect of the invention provides reagents e.g. antibodies, ligands, agonists and/or antagonists capable of specifically binding to ST2L for use in medicine, particularly therapy, prophylaxis or diagnosis.

Typically the reagent used for distinguishing and/or purifying Th1/Th2 cells may be an antibody specific for ST2L, more particularly specific for an epitope(s) located on the extracellular domain of ST2L. Preferably the antibody is raised against peptides of ST2L which have been judged (eg. by computer analysis) to possess substantially no cross-reactivity with other cellular proteins, such as IL-1 and IL-1 receptor superfamily.

Examples of such antibodies suitable for use in the present invention include antibodies raised against the following peptides of the mouse ST2L protein (numbering according to Yanagisawa et al (1993) FEBS 318, 83-87)

GEARIQEEGRNESSSNDMD; (residues 267-287)

YSDTNESIPTQKRNR; (residues 56-70)

SKSSWGLENEALIVR; (residues 27-41)

and in addition, antibodies raised against the following peptides of the human ST2 protein (numbering according to Tetsuka, T., et al (1992) [Biochim, Biophys. Acta 1171, 215-218]:

DFGEPRIQQEEGQNQSFSN; (residues 264-282)

RRHTVRLSRKNPSKE; (residues 312-327)

CLDMVLRIADVKEED; (residues 282-296)

YSTVSGSEKNSKIYC; (residues 118-133)

CKFIHNENGANYSVT; (residues 181-195)

CPRQGKPSYTVDWYYS; (residues 36-51)

SKQSWGLENEALIVRCP; (residues 26-42)

YSQTNKSIPTQERNR; (residues 56-70)

DLYNWTAPLEWFKNCQ; (residues 142-158)

The above peptides were identified on the basis of being on the extracellular portion of the ST2L protein, their hydrophilicity and for their uniqueness. The utility of antibodies raised against any of the above peptides may be verified by immunoabsorbance assay and/or by flow cytometry (for more specific details see the examples section).

Such antibodies may be polyclonal antibodies raised in a suitable host, such as a rabbit or monoclonal antibodies produced according to standard protocols, using spleen cells taken from mice immunised with a peptide and fusing with a suitable immortal cell line such as NSO myeloma

cells [See for example Golding J.W. (1986) Monoclonal Antibodies: Principles and Practice. Academic Press, London or Winter G. et al Trends in Pharmacol. Sci. 14: 139-143, 1993].

The term "antibody" as used herein is intended to include polyclonal or monoclonal antibodies or fragments thereof which are specifically reactive with murine and/or human ST2L or immunogenic fragments thereof. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example $F(ab')_2$ fragments can be generated by treating antibody with pepsin. The resulting $F(ab')_2$ fragment can be treated to reduce disulphide bridges to produce Fab' fragments. The antibodies described herein are further intended to include bispecific and chimeric molecules possessing an antigenic determinant to ST2L. The murine monoclonal antibodies may be humanised according to standard techniques [see for example Winter G. et al Trends in Pharmacol. Sci. 14: 139-143, 1993].

In one embodiment, a binding assay such as an immunoassay may be used to distinguish and/or identify Th1/Th2 cells. Many different immunoassay techniques are known to the skilled addressee. For example, wells of a microtitre plate (or other suitable solid substrate) can be coated with an antibody which specifically binds ST2L. After a suitable period of incubation with a sample comprising Th1/Th2 cells, Th2 cells will become bound to

the antibody, thereby forming an ST2L/antibody complex, thereafter unbound material (such as Th1 cells) may be removed by washing, and the amount of ST2L/antibody complex and consequently Th2 cells determined. The amount of ST2L/antibody complex may be determined by way of a further antibody specific for the anti-ST2L antibody. The further antibody may be suitably labelled, for example with a fluorescent label, a radiometric label or with an enzyme cleavable substrate, the product of which may be detected.

Both monoclonal and polyclonal antibodies directed against ST2L, and antibody fragments described above can be used as reagents to block ST2L function and allow study of, for example, Th cell proliferation or cytokine induction.

Antibodies which specifically bind ST2L epitopes can also be used in immunohistochemical staining of body fluid and tissue samples in order to evaluate the abundance or otherwise of Th2 cells. Anti-ST2L can be used diagnostically in flow cytometry to detect and evaluate Th2 levels in body fluid and/or tissue as part of a clinical testing procedure. By monitoring Th2 levels in an individual it is possible to determine the efficiency of a treatment given to an individual afflicted with a disorder associated with Th cell proliferation.

Antibodies of the present invention may be used in combination with complement in substantially selectively lysing Th2 cells in preference to Th1 cells. The antibodies alone, may also be used *in vitro* or *in vivo* to

inhibit Th2 cell function. Thus, the antibodies may be used to regulate the ratio of Th1:Th2 cells in a host organism or in an *in vitro* system. Controlling the ratio of Th1:Th2 cells may provide a means with which to control many infections and/or autoimmune diseases.

It is also possible to conduct drug screening programs for ailments in which Th1 or Th2 cells are implicated in the ailments, such as for afflictions associated with Th1 or Th2 cell proliferation or programs for identifying antagonists/agonists of ST2L. Such drug screening programs may be directed to drugs which may ameliorate the ailments and thereby reduce any associated Th1 or Th2 cell response. Alternatively drugs may have a direct action on the Th1 or Th2 cells and consequently cause a proliferation or diminution of Th1 or Th2 cells. Such drug screening may be carried out *in vitro* or *in vivo* and results determined by utilising the antibodies as described herein to ascertain the effect a particular drug has on the ailment by determining Th1/Th2 cell levels.

The antibodies of the present invention may themselves be used in therapy, as mentioned above, by controlling the relative proportions of Th1:Th2 cells in a host. Thus, the present invention also provides anti-ST2L antibodies for use in therapy and to the use of anti-ST2L antibodies in the manufacture of medicaments for therapy.

Since Th2 cells are the major mediators of allergic reactions, for example in asthma, reducing Th2 cell numbers and/or blocking cell functions via ST2L will be of

therapeutic value against such allergic reactions. Moreover, reducing Th2 cell numbers and/or blocking cell function via ST2L may result in a proliferation or perceived increase in the relative proportion of Th1 cells. Thus anti-ST2L antibodies are indicated as having application against diseases mediated by intra-cellular pathogens, such as leishmania and inflammatory diseases such as arthritis.

The above described uses may easily be adapted without undue burden by the skilled addressee to Tc1/Tc2 cells also.

The present invention will now be further described by way of example with reference to the following methods and examples section.

METHODS

Cell lines and tissue culture. Dorris (specific for egg lysozyme) and D10 (specific for conalbumin) were obtained from ATCC (Atlanta, Georgia). X4, X8, X9, X12, X17, Y8, D2.2 and D2.3 were cloned and maintained as described previously in Rossiter, B.A., et al (1994) Eur. J. Immunol. 24, 1244-1247 (specific against group A streptococcal M protein). The cell lines were maintained by periodic antigen stimulation with irradiated antigen presenting cells followed by expansion in medium containing IL-2. For short-term cultured cell lines, spleen cells from D011.10 mice were purified for $CD4^+$ T cell by negative selection as described previously by Openshaw, P. et al.

(1995) J. Exp. Med. 182, 1357-1367. The cell lines were cultured with OVA peptide ($OVA_{323-339}$) and irradiated BALB/c spleen cells in the presence of IL-12 plus anti-IL-4 (for Th1 cell line) or IL-4 (for Th2 cell line) for 6 days.

Differential display-polymerase chain reaction (DD-PCR). DD-PCR was performed according to Liang, P. and Pardee, A.B. (1992) Science 257, p967-971 using RNAimage kit (GenHunter corporation, Brookline, MA02146, USA). Total RNA was treated with DNase I (GenHunter Corp.) to remove any trace of DNA. Purified RNA (1 μg) was transcribed into cDNA using MMLV reverse transcriptase. The cDNA was amplified by using AmpliTaq DNA polymerase (Perkin Elmer) with 50 pairs of primers (GenHunter). PCR reactions were performed in the presence of [^{35}S]-dATP (NEN, Boston, USA), and products separated in 6% sequencing gels under denaturing conditions. DNA fragments from Th1 and Th2 cells were then compared. Differentially expressed distinct bands longer than 150 bp were excised from the dried gel, extracted by boiling in 100 μl of dH_2O, and precipitated with ethanol in the presence of glycogen. The DNA was further amplified with the original set of primers used to generate the particular band, using the same thermal cycle conditions. PCR products were electrophoresed in a 1.5% agarose ethidium bromide gel to confirm the band size before cloning into PCRII vector (InVitrogen) and sequenced using M13R and M13(-20)F primers. (available from GenHunter Corp. USA).

Reverse Northern blot. This was used to screen and confirm the differentially expressed mRNA according to the method described in Zhang, H., et al (1996) Nucl. Acid Res. 24, 2454-2456 using a ReversePrime Kit (GenHunter Corp.). Denatured PCR products (100 ng) were dot-blotted onto duplicate nylon membranes by the slot blot filtration manifolds (Hoefer, San Francisco, CA94107), using murine IL-5 and β-actin cDNA as control and equal loading. Membranes were UV cross-linked and hybridised with equal amounts to Th1 or Th2 cDNA probes (10^6 cpm). The probes were prepared from 10-50 μg of RNA from each of the cloned Th1 or Th2 cells by reverse transcription, using 1000 units of MMLV reverse transcriptase in the presence of α-[^{32}P]dCTP and T20 primer. (available from GenHunter Corp. USA). The hybridisation patterns of duplicate membranes were then compared.

Northern blot. Total RNA was isolated from cells by RNAZolB (available from Biogenesis Ltd. Poole, UK) and separated (20 μg/lane) in 1% formaldehyde agarose gels, blotted onto a nylon membrane and hybridised with a 3' terminal cDNA fragment of ST2L, labelled with α-[^{32}P]dATP. The 3' cDNA fragment was cloned from D10 cells by DD-PCR using the primer pair: AAGCTTTTTTTTTTA and AAGCTTCAGCAGC (designated H-T11A and H-AP34 respectively, GenHunter Corp.).

RT-PCR Southern Blot. Primers for amplifying ST2L were: sense, ACTTTGTTCACCACACTCTGC; antisense, AACAGATGCCGTCTTGGAGGC (which generate a product of about 450 bp). Primers for β-actin were: sense, GTGGGCCGCTCTAGGCACCAA; antisense, CTCTTTGATGTCACGCACGATTTC (which generate a product of about 540 bp). PCR products were separated in 1% agarose gels. Membranes were hybridised with internal probes (ST2L: ATTGAAATGGAGCCTCTGGGTGAGGCAAGC; β-actin: CAGAGCAAGAGAGGTATCCTGACCC) labelled with γ-[^{32}P]dATP using T4 polynucleotide kinase.

Antibody to ST2L. A rabbit was immunised with the peptide corresponding to residues 267-287 of murine ST2L (numbering according to Yanagisawa et al (1993) FEBS Letters 258, 83-87). The peptide was conjugated to keyhole limpet haemocyanin (KLH). The rabbit was injected with 100 μg of peptide conjugated to KLH in Freund's incomplete adjuvant subcutomeously. The rabbit was boosted four weeks later with the same antigen in incomplete adjuvant. The rabbit was further boosted with an iv injection of 100 μg of the antigen in saline. The rabbit was bled out one week later. The peptide was selected to have no homology with IL-1 receptor and within the extracellular region of ST2L. Specificity of the antibody was tested by Western blot against recombinant ST2L and by ELISA with peptide-bound 96-well plates. Total IgG was purified from immune serum and pre-immunised serum by protein A column or by Annonium

sulphate precipitation according to standard protocols. Purity of the IgG was >95% as determined by SDS-PAGE coomasse blue staining.

A further rabbit was immunised with a peptide corresponding to residues 264-282 of human ST2 (numbering according to Tetsuka, T., et al (1992) Biochim. Biophys. Acta 1171, 215-218) according to the same protocol. Additionally a monoclonal antibody was raised against the same peptide and prepared according to the protocol as set out in Winter et al 1993 [Trends in Pharmacol. Sci. 14: 139-143].

Flow cytometric analysis. Cells were incubated with anti-murine ST2L antibody (40 μg/ml) followed by flouresceine isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (SAPU, Scotland). All reactions and washing were carried out at 4°C in phosphate buffered saline (PBS) containing foetal calf serum (FCS) (5%) and NaN_3 (0.1%), and all antibodies were centrifuged (11,000xg, 5 min) immediately before use. For three colour staining, cells were suspended at 10^5-10^6/ml and stimulated with phorbol myristate acetate (PMA, 50 ng/ml, Calbiochem) plus ionomycin (500 ng/ml, Calbiochem) for 4h. Brefeldin A (10 μg/ml, Sigma) was added during the last 2 h. Cells were then incubated with anti-ST2L antibody or control IgG followed by biotinylated goat anti-rabbit IgG (DAKO) and then developed with PerCP-streptavidin (Becton Dickinson, USA). All incubations and washing were carried out at 4°C

in PBS containing FCS (5%) and NaN_3 (0.1%). The cells were then fixed with paraformaldehyde (2%), and, after washing, permeabilised at room temperature with 0.5% saponin (Sigma) in PBS and FCS (5%), and stained sequentially with FITC-conjugated anti-murine IFN-γ (IgG2a, PharMingen) and phycoerythrin (PE)-conjugated anti-murine IL-4 (IgG2a, PharMingen), or FITC- and PE- conjugated isotype control antibodies (Becton Dickinson). Samples were analysed on a FACScan flow cyometer (Becton Dickinson).

Complement-mediated cell lysis. Cloned T cell lines (see Table 1) ($1x10^6$ cells in 0.2 ml culture medium) were incubated with various concentrations of anti-ST2L antibody or normal rabbit IgG (clarified by centrifugation) for 30 min on ice. The cells were washed once and then incubated with guinea pig complement (1/10 dilution, Harlan sera-lab, UK) for 45 min at 37°C. Cell viability was estimated microscopically (counting >500 cells) by staining with acridine orange and ethidium bromide according to standard protocols.

Leishmania infection. BALB/c mice (female, 6-8 weeks old, Harlan Olac, Bicester, UK) were infected in the right hind footpad with $1x10^6$ stationary phase promastigotes of *Leishmania major* (LV39) and injected intra peritoneally (ip) with 250 μg of rabbit anti-ST2L antibody or normal rabbit Ig. The antibody was administered for the next two days, and then every 3-4 days for 1 week followed by weekly

injections. Lesion development was followed by measuring the footpad swelling (difference between the infected right and uninfected left hind footpad). The maintenance of parasites, infection, and measurement of disease progression were as described previously by Howard et al (1980) [J. Exp. Med. 152, 594-607]. At the end of the experiment, mice were killed by cervical dislocation. Footpad was removed for assay of parasite load by limiting dilution according to McSorley et al (1997) [Infect. Immun. 65, p171-178]. Draining lymph node cells were harvested and cultured ($1x10^6$/ml) *in vitro* with frozen and thawed parasite antigen (10^5-10^7 parasite/ml equivalent). Culture supernatant was harvested at 48 h and assayed for cytokines by ELISA using paired antibodies (PharMingen). Anti-leishmanial specific antibody in the serum was titrated for IgG1 and IgG2a as described previously by McSorley et al (1997).

Induction of collagen induced arthritis (CIA) in mice. Male DBA/1 mice (6-8 weeks old, Harlan Olac, UK) were injected intradermally with 200 μg of bovine type II collagen (Sigma) in Freund's complete adjuvant (d-21). Collagen (200 μg in PBS) was given again on d0 by ip injection. Daily injections (ip, 200 μg/mouse) of anti-ST2L antibody or normal rabbit IgG were administered from d1 for 5 days. Mice were monitored daily for signs of arthritis for which severity scores were derived as follows: 0 - normal, 1 - erythema, 2 - erythema +

swelling, 3 - extension/loss function, total score - sum of 4 limbs. Paw thickness was measured with a dial-calliper (Kroeplin, Germany). Quantification of arthritis was performed as previously described [Joosten et al (1997), Arthritis Rheum, 40, 249-260].

Example 1 Differential expression in Th1 and Th2 cells

Total RNA from a panel of four of each cloned Th1 (Dorris, X4, X9 & X17) and Th2 (D10, D2.2, D2.3 & X12) cell lines were compared by DD-PCR. Using 50 primer pairs, more than 50 distinct bands were detected, of which 10 fragments have so far been confirmed to be differentially expressed in Th1 or Th2 cells using reverse Northern blot [Zhang, H. et al (1996) Nucl. Acid. Res. 24, 2454-2456] and Northern blot (Two representative patterns comparing the PCR products from Dorris (Th1) and D10 (Th2) cells, using two different primer pairs (lanes 1 & 2, H-T11A/H-AP34; lane 3 & 4, H-T11G/H-AP34) are shown in Fig. 1a. Arrow indicates the ST2L band as determined by nucleotide sequencing and comparison with nucleic acid databases. Northern blot analysis demonstrated the constitutive expression of ST2L in all four Th2 but not in Th1 clones tested (see Fig. 1b). The more sensitive reverse transcription PCR Southern blot also failed to detect ST2L message in a panel of Th1 clones. In contrast, the message was readily demonstrated in four Th2 clones and two Th0 clones (which produce IFN-γ, IL-4 and IL-5) (see Fig. 1c).

Example 2 Regulation of ST2L expression

The regulation of ST2L expression in Th clones was analysed *in vitro* by various immunological stimuli. Representative Th1 (X9) and Th2 (D10) clones were stimulated for 3, 6 or 12 h with: (see figure 2a) Lane 1, Con A (2.5 μg/ml); lane 2, IL-1β; lane 3, IL-5; lane 4, IL-2; lane 5, IL-4; lane 6, IFN-γ (all cytokines were recombinant murine proteins obtained from Genzyme used at 5 ng/ml); lane 7 anti-IFN-γ (1 μg/ml); lane 8, anti-IL-4 (1 μg/ml); lane 9 medium alone; lane 10, anti-CD3 (1.5 μg/ml). Results for 12 h (not shown) were the same as for 3 h, except no message was detected in X9. D10 cells expressed ST2L constitutively and stably, whereas X9 cells failed to do so, except a weak and transient response at 3 h and 6 h to IL-5 and anti-CD3 respectively.

In contrast to Th2 clones which expressed ST2L constitutively, Th1 clones did not express ST2L even when stimulated with concanavalan A (Con A), IL-1β, IL-2, IL-4, IFN-γ, or with anti-IFN-γ or anti-IL-4 antibodies. Following incubation with IL-5 or anti-CD3 antibody, Th1 clones expressed a weak ST2L message transiently at 3 and 6 h post-activation respectively (Fig. 2a); but this disappeared completely by 12 h. The expression of ST2L in Th2 cells was not affected by these stimuli (Fig. 2a).

As ST2L is a delayed, early proliferative response gene in BALB/c-3T3 cells induced by serum factors [Yanagisawa, K., et al (1993) FEBS 318, 83-87], the regulation of ST2L expression in Th clones by serum was examined. Cells were

rested for 48 h in serum-free medium and then cultured in medium containing 20% FCS. The expression of ST2L on Th2 clones was diminished when cultured in serum-free medium, but restored soon after the addition of serum. The expression of ST2L in Th0 clones was not affected by serum starvation. In contrast, no ST2L message was detectable in Th1 clones before or 6 h after addition of serum (see Fig. 2b: X9 (Th1, lane 1), X4 (Th1, lane 2), D10 (Th2, lane 3) and Y8 (Th0, lane 4).

Example 3 Localization of ST2L

To study the location and function of the molecule, a polyclonal rabbit antibody against a peptide, corresponding to the extra-cellular domain of ST2L, was raised and FACS analysis of cell surface expression of ST2L carried out. Th1 (X4) and Th2 (X12) cells were stained with rabbit anti-ST2L antibody (green) or preimmune rabbit IgG (white) (40 μg/ml) and developed with a FITC-conjugated donkey anti-rabbit IgG. Stable cell surface staining of Th2 clones but not Th1 clones was detected with this antibody (Fig. 3a). Similar results were obtained for three other Th1 and Th2 clones (not shown). Th0 (Y8, data not shown) cells had the same staining pattern as Th2 cells. The staining of Th2 cells (X12) with anti-ST2L antibody can also be visualised by fluorescent microscopy (x400). Staining can be blocked by the ST2L peptide (100 μg/ml) and no staining was detected on Th1 cells (data not shown).

PMA and ionomycin-activated Th2 (X12) and Th1 (X4) clones were stained for cell surface ST2L (with PerCP), followed by intra-cellular staining with anti-IL-4 (with PE) and anti-IFN-γ (with FITC). Cytokines in the culture supernatants (48 h) of T cell clones ($1x10^6$/ml) stimulated with specific antigen and irradiated antigen presenting cells were (IFN-γ vs IL-4, ng/ml): X4, 5.2 vs <0.02; X12, <0.01 vs 0.25. Single cell, three colour flow cytometric analysis demonstrated that ST2L co-expressed with IL-4 but not with IFN-γ in cloned T cell lines (Fig. 3b). This staining pattern also occurred in cells derived from naive T cells.

Splenic $CD4^+$ cells from the ovalbumin T cell receptor (OVA TCR)-αβ transgenic mice (DO11.10) [Murphy, K.M., et al (1990) Science 250, 1720-1723] were driven to the Th1 or the Th2 lineage *in vitro* in a 6 day culture. The cells were then stimulated and stained as described above. Cytokines in the culture supernatants (day 3) were (IFN-γ vs IL-5, ng/ml): Th1 line, 2.6 vs <0.02; Th2 line, <0.01 vs 0.52. Th1 cells did not express ST2L or IL-4, but showed intra-cellular staining for IFN-γ. In contrast, Th2 cells expressed ST2L and IL-4, but produced no detectable IFN-γ (Fig. 3c).

Moreover, the antibody was also shown to strongly lyse Th2 cells but had only a modest effect on Th1 cells *in vitro* in the presence of complement (see Table 1).

Additional experiments were performed to ascertain whether the anti-ST2L antibody can also distinguish $CD8^+$

cells into two distinct subsets (called cytotoxic T cell type 1 and 2, or Tc1 and Tc2). Spleen cells from C57BL/6 mice bearing TCR transgene specific for peptide 257-264 of ovalbumin (called OT-I cells) [Hogquist KA et al. *Cell* 76(1) 17-27 (1994)] were driven to the Tc1 or Tc2 lineage *in vitro* in a 6 day culture as described for Th1 and Th2 above. The cells were then harvested, stimulated and stained as above. While Tc2 cells were strongly positive [Fig. 3d(a)], Tc1 cells were only weakly stained [Fig. 3d(b)].

Example 4 The effect of anti-ST2L antibody *in vivo*

BALB/c mice are genetically highly susceptible to *Leishmania major* infection [Liew, F.Y. et al (1993) Leishmania. Adv. Parasitol. 32, 161-259]. Disease progression and resistance are associated with the preferential development of Th2 or Th1 cells, respectively [Reiner, S.L. et al (1995) Ann. Rev. Immunol. 13, 151-177]. Fig. 4 demonstrates that BALB/c mice infected with *Leishmania major* compared with controls, n=10 and treated with anti-ST2L antibody developed significantly smaller lesions and 3 orders of magnitude less parasite load compared with controls receiving normal rabbit IgG (Fig. 4a). Draining lymph node cells (n=10) from the anti-ST2L-treated mice produced significantly more IFN-γ, but less IL-4 and IL-5 compared with cells from control IgG-treated mice (Fig. 4b). IL-12 levels were not affected. Serum from anti-ST2L-treated mice produced markedly more IgG2a

and similar amounts of IgG1 leishmanial-specific antibody compared with serum from the control mice (Fig. 4c).

Collagen-induced arthritis (CIA) in susceptible DBA/1 mice is a predominantly Th1-mediated disease [see Simon, A.K. et al (1994) Proc. Natl. Acad. Sci. USA 91, 8562-8566], the immunopathogenesis of which closely resembles clinical rheumatoid arthritis [see Trentham D.E. et al (1977) J. Exp. Med. 146, 857-868; Courtenay, J.S. et al (1980) Nature (London) 283, 666-668]. The present inventors therefore examined whether administration of anti-ST2L antibody could modify CIA. Incidence and severity of disease development were markedly enhanced in mice which received anti-ST2L antibody, compared with controls injected with normal IgG [Fig. 5a-c (a) Incidence rate, (b) clinical score, and (c) number of arthritic paws. Data are mean ± SEM, n=15[. Mice (n=4) were sacrificed 48 h after antibody treatment and spleen cells were pooled and cultured with collagen (50 μg/ml) for 48-96 h, and culture supernatants assayed for cytokines by ELISA. T cell proliferation was expressed as [^{3}H]thymidine uptake in triplicate cultures (mean cpm x 10_3 ± SEM): antibody-treated, 52.8 ± 6.8; control, 27.8 ± 1.1 ($p<0.05$).

On stimulation with collagen *in vitro*, spleen cells from the antibody-treated mice also produced significantly more IFN-γ and IL-6 compared with cells from control mice (Fig. 5d). IL-4 and IL-5 were not detected (<20 pg/ml).

The anti-ST2L antibody also markedly exacerbated LPS-induced mortality in BALB/c mice (Table 2). This was accompanied by elevated concentrations of serum IFN-γ, tumour necrosis factor (TNFα) and IL-6.

Since Asthma is known to be a Th2 driven disease, the effect of anti-ST2L antibody on experimental asthma in the murine model was investigated *in vivo*. Mice (female BALB/c, 8 weeks old from Harlan Ltd, UK) were immunised subcutaneously with 2 μg of the house dust mite antigen, *Der p1* (from ALK, Horsholm, Denmark) in aluminium hydroxide (from Pierce and Warriner, Chester, UK) as adjuvant on day 0. Mice were injected intraperitoneally with the anti-ST2L antibody (250 μg/mouse/day in PBS) or normal IgG on day -1, 0, 1,4,8,11, 15, 18, 22 and 25. They were challenged intranasally with *Der p1* antigen (20 μg/mouse in PBS) on day 25. All the mice were sacrificed on day 27 and lung pathology examined. The antibody treated mice developed markedly reduced lung eosinophilia compared with the control IgG-treated mice (Figure 6, $p<0.01$, $n=10$). These results clearly indicate that the anti-ST2L antibody is effective in reducing the lung pathology in house dust mite-induced experimental asthma, and therefore may be of use in the treatment of clinical asthma.

Monoclonal anti-ST2L antibody has now been developed. This has been achieved by a standard hybridoma technique (see p7). Spleen cells from a rat immunised with the ST2L peptide were fused with NSO cells. Anti-ST2L producing clones were then screened by ELISA and by FACS analysis

using cloned Th2 cells. Monoclonal antibody was then produced in bulk tissue culture.

Rabbit anti-human ST2L polyclonal antibody was also produced. A rabbit was immunised with a peptide corresponding to human ST2L (see p6, residues 264-282) according to the protocol detailed on p13. The antibody stains human Th2 but not Th1 cells.

Table 1. Effect of anti-ST2L antibody on T cells *in vitro*

Treatment of cells	**Th1 (X4)**	**Th2 (X12)**
	(% viability)	
Anti-ST2L + C'	75.2±2.1*	9.7±0.5**
Anti-ST2L alone	94.9±3.5	95.2±4.6
Normal IgG + C'	95.9±2.7	94.6±3.3
Normal IgG alone	95.0±2.9	97.2±5.8
Medium alone	96.2±3.5	95.8±3.9

Th1 and Th2 cells were incubated with 400 μg/ml of antibody and then with or without complement (C'). The cell viability was determine microscopically. At this concentration, in the absence of complement, anti-ST2L antibody, but not Normal IgG, also partially suppressed the proliferation and cytokines production by Th2 cells *in vitro* in response to specific antigen and antigen presenting cells. The antibody did not affect the proliferation of Th1 cells (data not shown). Data are mean

± SEM, n=3. *p<0.05, **p<0.001 compared with medium control.

Table 2. Anti-ST2L antibody increased LPS-induced mortality.

Antibody Treatment	Cytokines in serum (pg/ml) IFN-γ	TNFα	IL-6	Cumulative mortality (72 h)
Normal IgG	7265±717	271±33	9235±848	6/15
Anti-ST2L	15803±767**	409±16*	18803±2568**	13/15**

BALB/c mice were injected ip with 18 mg/kg of LPS on day 0. They were also injected ip daily with 400 μg/mouse of antibody or normal IgG from day -1. Serum were obtained from tail vein 2 h after LPS injection and pooled for cytokine assays by ELISA. Experiments were terminated at 72 h according to the UK Home Office guideline. Two sample T-test was used for cytokine comparisons and two-tail log-rank test for survival analysis between the two groups of mice. *p<0.05; **p<0.02. There was no difference in the IL-10 or IL-12 concentrations between the two groups, IL-4 and IL-5 were not detected (<40 pg/ml). Results are representative of 2 experiments.

CLAIMS

1. An antibody specific for an epitope(s) located on the extracellular domain of ST2L.

2. The antibody according to claim 1 which has been raised against a peptide of ST2L which has been judged to possess substantially no cross-reactivity with other cellular proteins.

3. The antibody according to claim 2 which is specific for mouse ST2L and has been raised against a peptide selected from the group consisting of:
GEARIQEEGRNESSSNDMD;
YSDTNESIPTQKRNR; and
SKSSWGLENEALIVR.

4. The antibody according to claim 2 which is specific for human ST2L and has been raised against a peptide selected from the group consisting of:
DFGEPRIQQEEGQNQSFSN;
RRHTVRLSRKNPSKE;
CLDMVLRIADVKEED;
YSTVSGSEKNSKIYC;
CKFIHNENGANYSVT;
CPRQGKPSYTVDWYYS;
SKQSWGLENEALIVRCP;
YSQTNKSIPTQERNR; and

DLYNWTAPLEWFKNCQ;

5. The antibody according to any preceding claim wherein the antibody is a monoclonal antibody.

6. The monoclonal antibody according to claim 5 wherein the monoclonal antibody has been humanised.

7. Use of an antibody according to any preceding claim in a binding assay.

8. Use according to claim 7 wherein the assay is used to distinguish and/or identify Th1/Th2 cells.

9. Use according to claim 7 wherein the assay is a diagnostic assay for evaluating the abundance or otherwise of Th2 cells in a sample of body fluid or tissue.

10. Use of an antibody according to any one of claims 1 to 6 in combination with complement to substantially selectively lyse Th2 cells in preference to Th1 cells.

11. Use of an antibody according to any one of claims 1 to 6 to inhibit Th2 cell function.

12. Use of an antibody according to any one of claims 1 to 6 in the manufacture of a medicament for use in therapy.

13. Use according to claim 12 wherein the medicament is intended for use as a mediator of an allergic reaction.

14. Use according to claim 13 wherein the allergic reaction is asthma.

15. Use according to claim 12 wherein the medicament is intended for use against diseases mediated by intra-cellular pathogens.

16. Use according to claim 15 wherein the intra-cellular pathogen is Leishmania.

17. Use according to claim 12 wherein the medicament is intended for use against an inflammatory disease.

18. Use according to claim 17 wherein the inflammatory disease is arthritis.

19. A method for distinguishing cells which express membrane bound ST2L from cells that do not, comprising contacting a sample of cells with a reagent specific for ST2L and determining the presence or absence of an ST2L reagent complex.

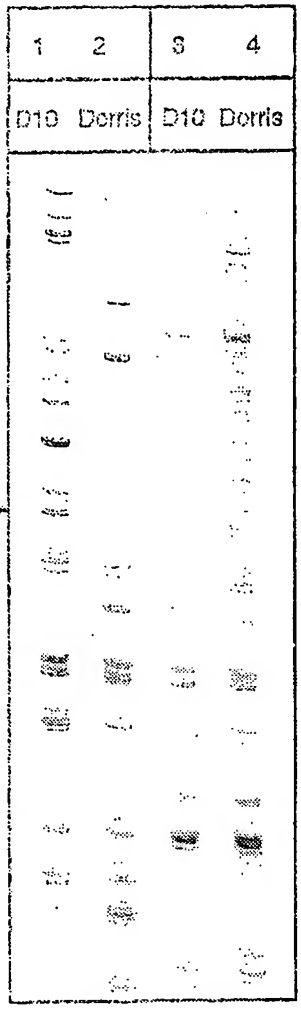
20. The method according to claim 19 wherein the cells which express membrane bound ST2L are Th2 cells.

21. A method of purifying Th1 or Th2 cells from a sample comprising Th1 and/or Th2 cells, the method comprising contacting the sample with a reagent specific for ST2L, such that any Th2 cells present in the sample bind to the reagent, and removing any unbound material.

22. A reagent for use in the method according to any one of claims 19 - 21 wherein the reagent is selected from antibodies, ligands, agonists and/or antagonists capable of specifically binding to ST2L.

23. Antibodies, ligands, agonists and/or antagonists capable of specifically binding to ST2L for use in medicine.

a



b

4401 ACAACAGCAA CAGCTGCAGC AGCAACAACA ACAACAAAGA AAAAGAAGAG

4451 GAGGAGGAGC AAAGGAAAGA AGGAAGAAGG AAGAAGAAAG GGAAGAAATA

4501 ATAGATTTTT CTGTAATGAA CACACATATG CTTTGATGCT TTTGCTAAAC

4551 TCAAAATATT AGTTTTATTT TACTGTTTTG AAAGGTTCAA AGCATGATCC

4601 ATGTAAAAAT GTCTTCTGTG GGGCTTTCTC CCATTTCTAC TTTTGTTCCC

4651 CTCATTTCTT CAAAGTGCTT GTCCAGGCAG AGCTGACCTT ATTTGCTTAC

4701 CAGTTACAGG TAAACAAAGC GTTTCCTCGT GTTGCCTCTT GTAGCCATCT

4751 CTGTATTAGA TTAGGAAGGG AAGGAGCCGT CCTACTGTCC AGTTTGTGAG

4801 TTCTGGTAGA AAGAGTGTTG AAGTTTGTTA AATGCTTGTT TTCCATGTAT

4851 CAAAATGTTA TGCCTTTCCT ATTTATTATT GTATGACAAA TTATTTTTCA

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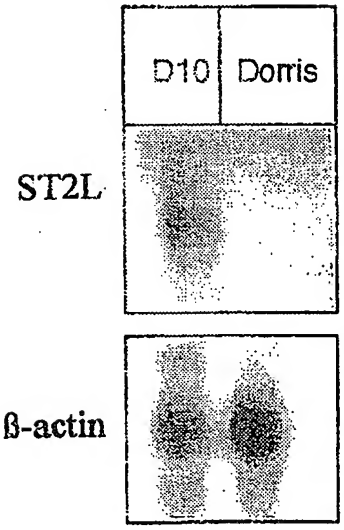
4951 TGTTTGAAGA GTTCTAGCTT ATTAAAAAAA AAAATCTAG

c



d

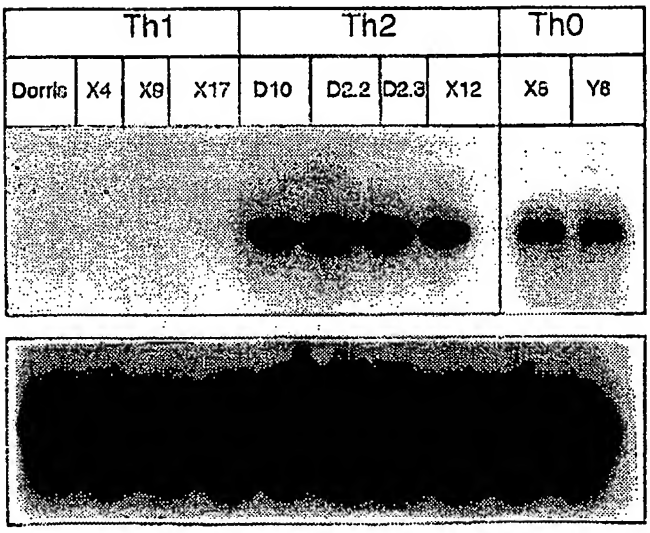


Fig. 1

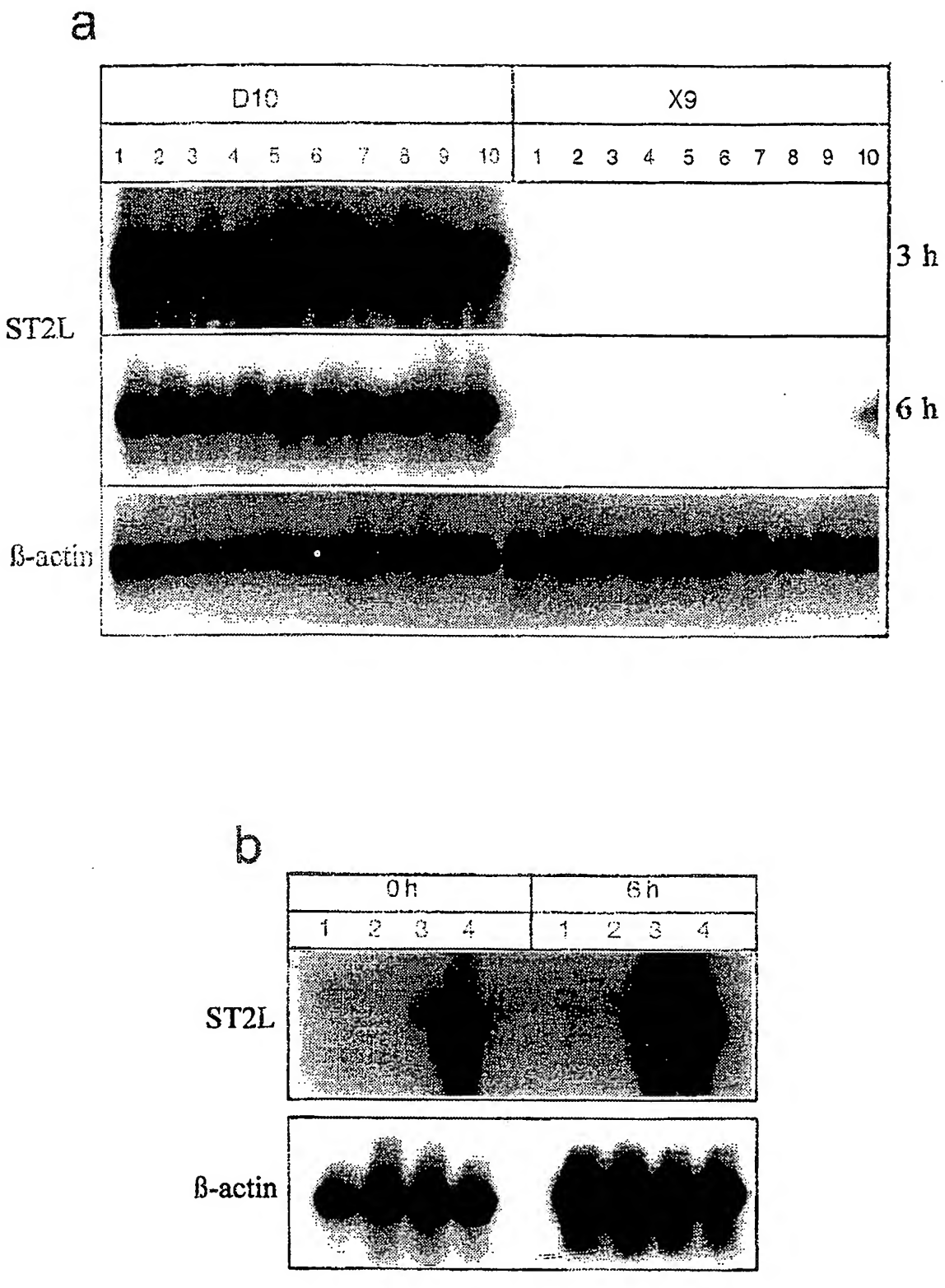
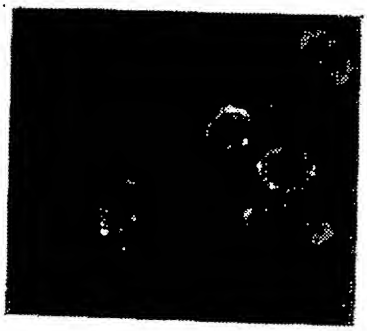

a
D10
X9
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
3 h
6 h
ST2L
ß-actin
b
0 h
6 h
1 2 3 4
1 2 3 4
ST2L
ß-actin

Fig. 2



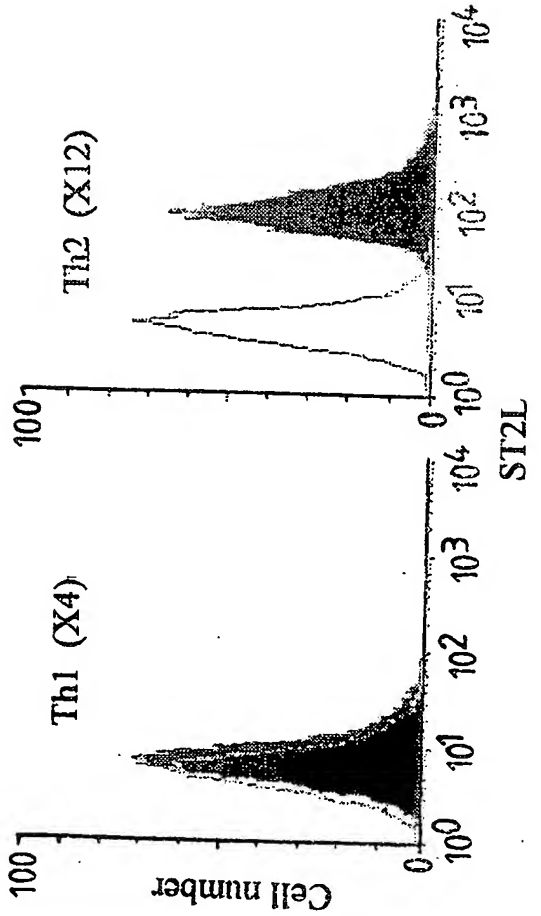


Fig. 3a

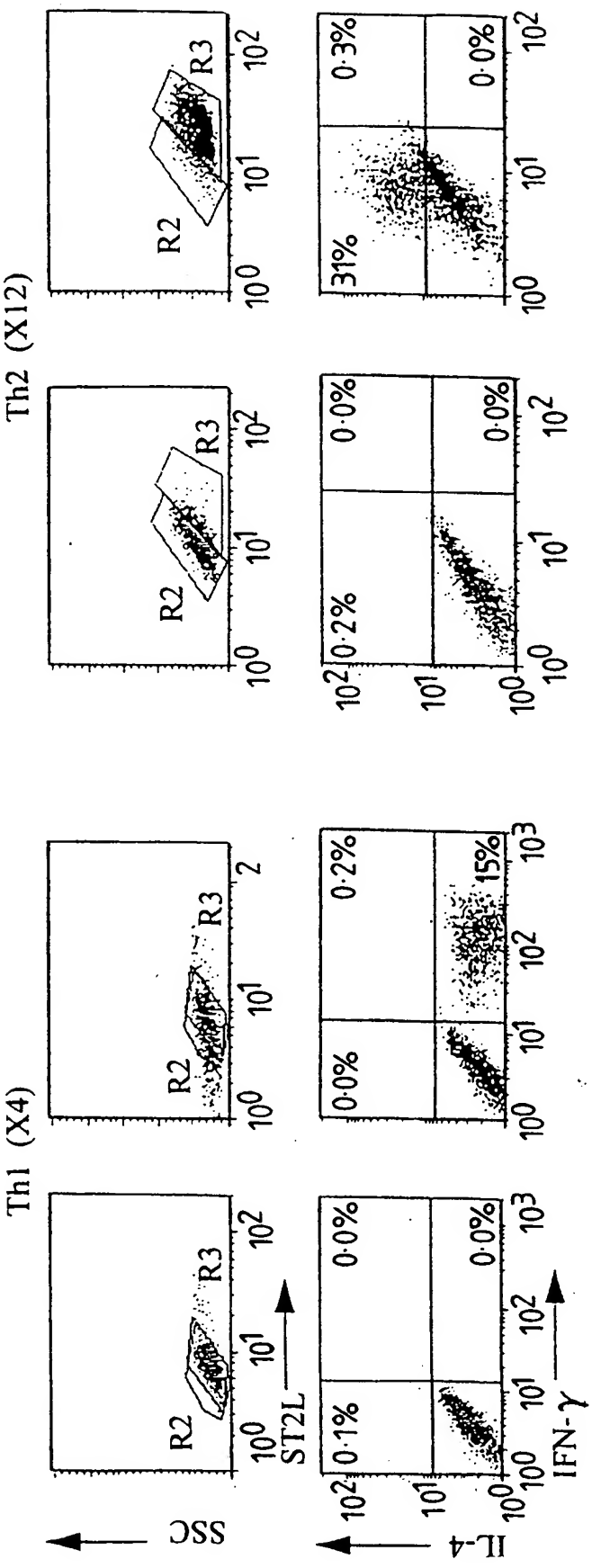


Fig. 3b

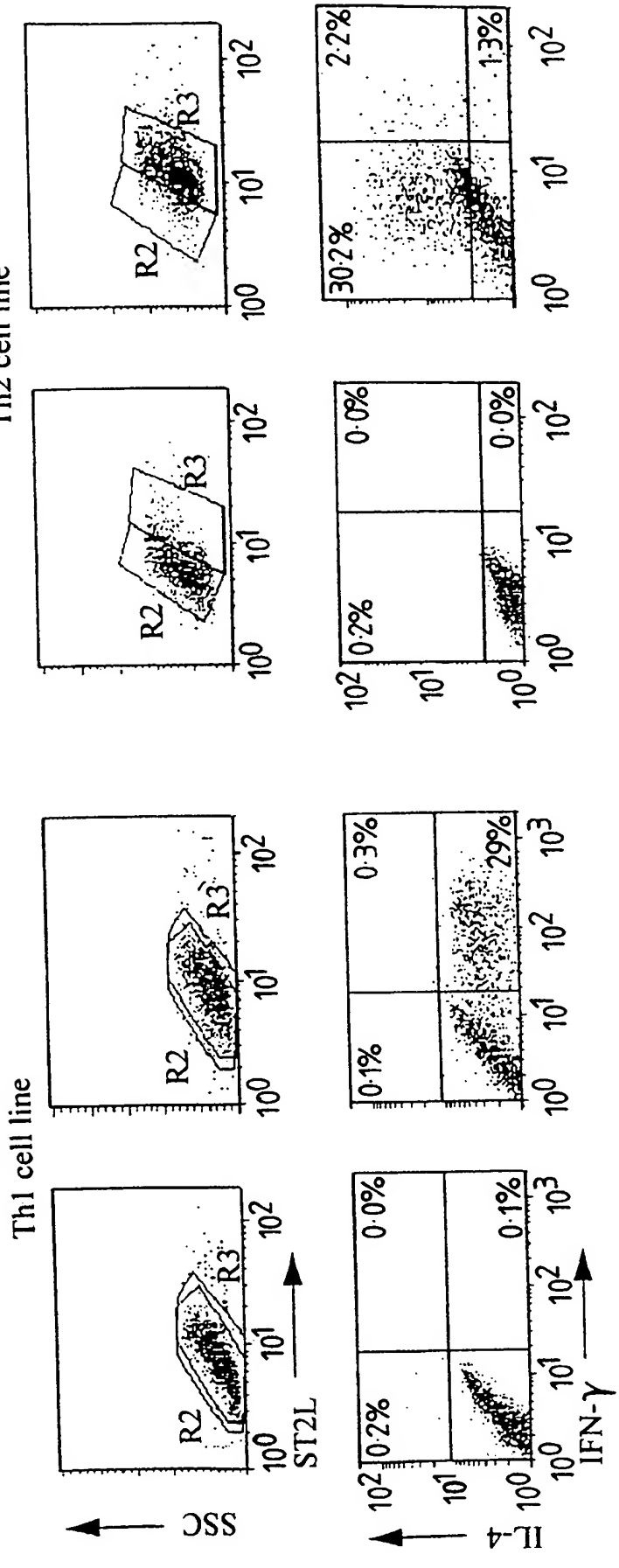

Th1 cell line
Th2 cell line
SSC
ST2L
IL-4
IFN-γ
R2
R3
0·0%
0·2%
0·1%
29%
0·3%
30·2%
2·2%
1·3%

Fig. 3c

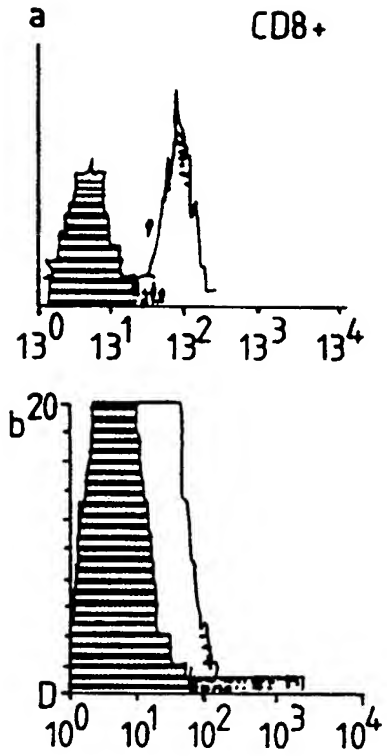


Fig. 3d

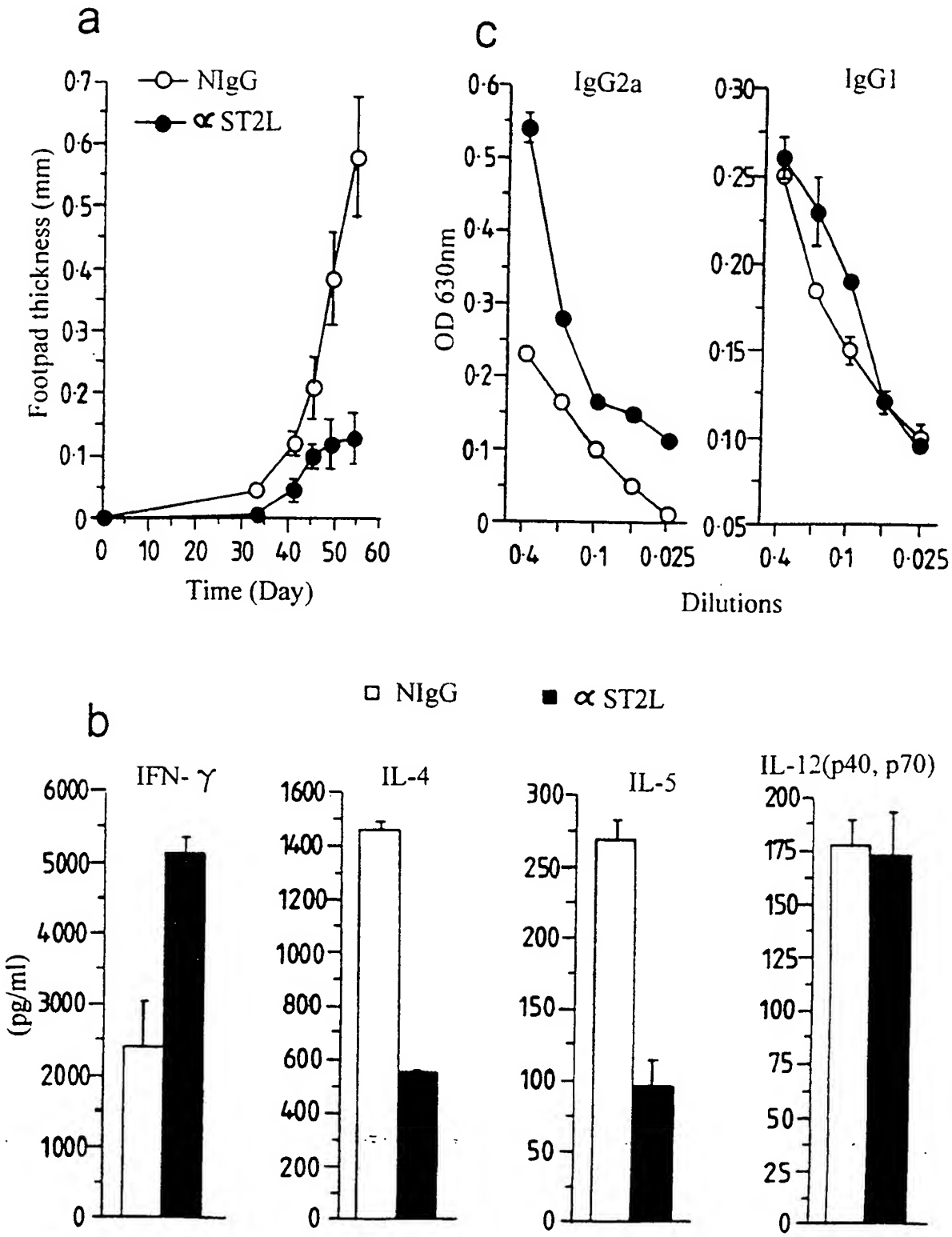

a
NIgG
∝ ST2L
Footpad thickness (mm)
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
0 10 20 30 40 50 60
Time (Day)
c
IgG2a
IgG1
OD 630nm
0·6
0·5
0·4
0·3
0·2
0·1
0
0·4 0·1 0·025
0·30
0·25
0·20
0·15
0·10
0·05
0·4 0·1 0·025
Dilutions
b
NIgG
∝ ST2L
IFN-γ
IL-4
IL-5
IL-12(p40, p70)
(pg/ml)
6000
5000
4000
3000
2000
1000
0
1600
1400
1200
1000
800
600
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Fig. 4

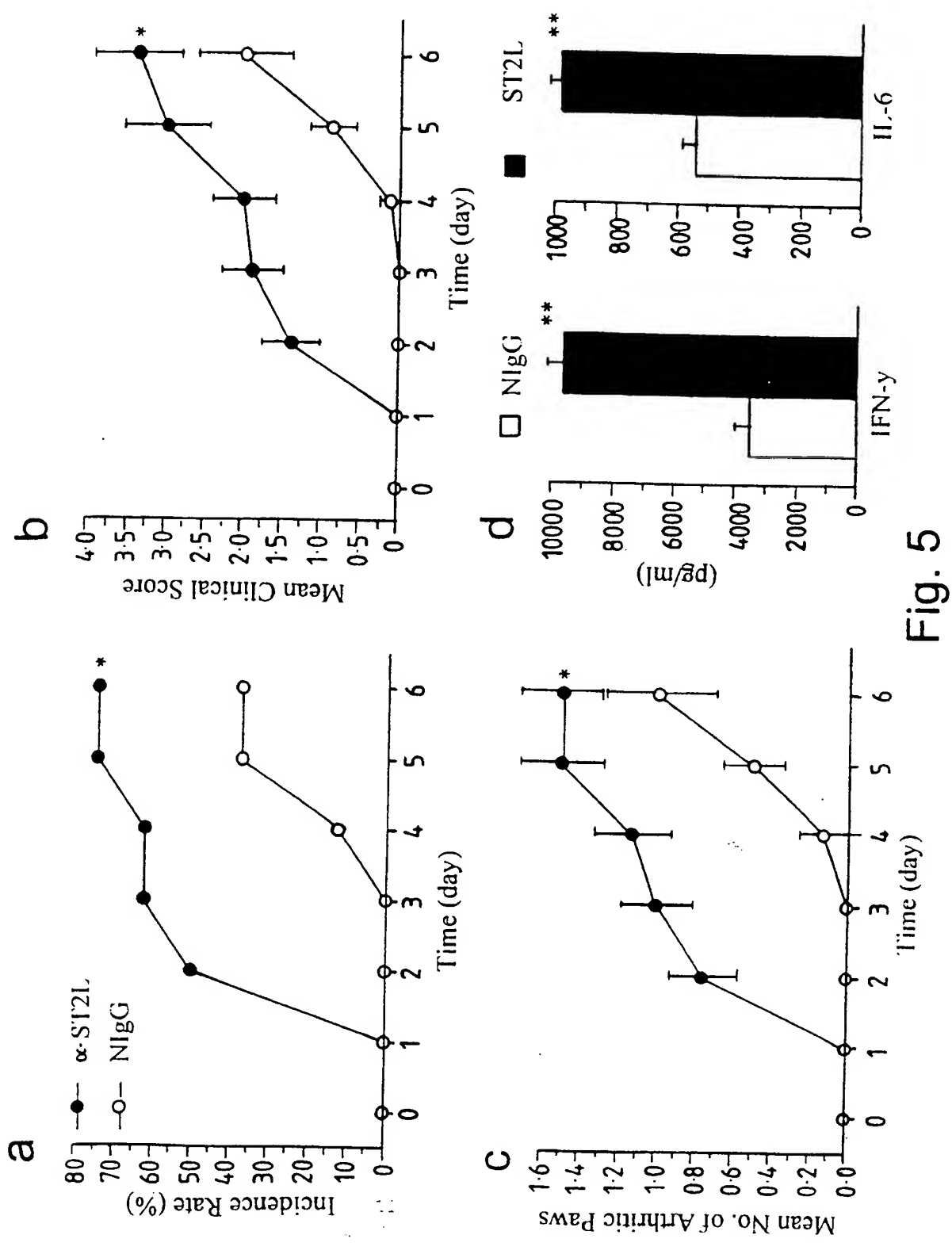
a
α-ST2L
NIgG
Incidence Rate (%)
Time (day)
b
Mean Clinical Score
Time (day)
c
Mean No. of Arthritic Paws
Time (day)
d
NIgG
ST2L
(pg/ml)
IFN-γ
IL-6

Fig. 5

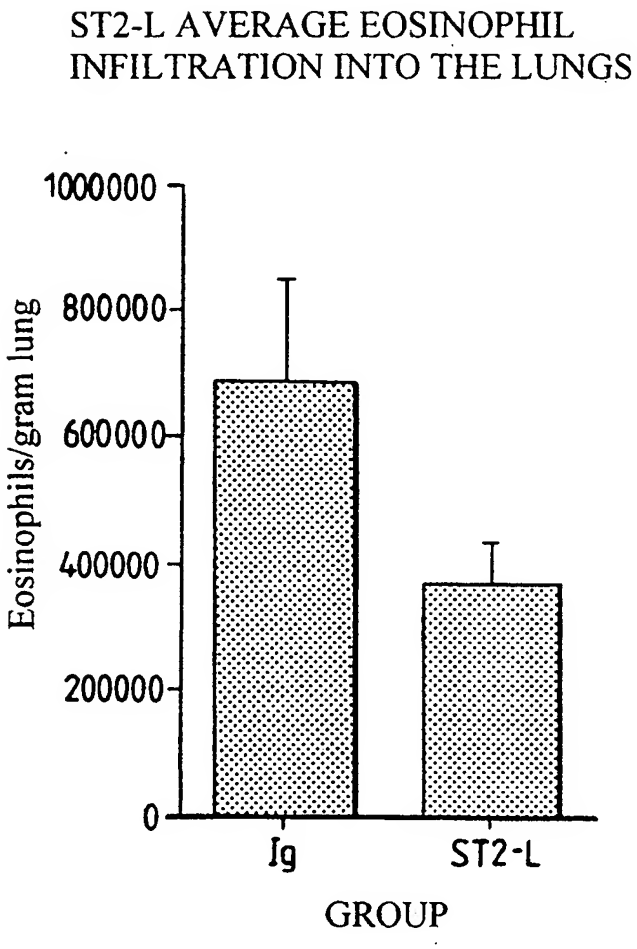
ST2-L AVERAGE EOSINOPHIL
INFILTRATION INTO THE LUNGS
Eosinophils/gram lung
1000000
800000
600000
400000
200000
0
Ig
ST2-L
GROUP

Fig. 6

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/GB 98/03913

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/569 A61K39/395 C07K16/28 //(A61K39/395,38:17)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YOSHIDA K. ET AL.: "Studies on natural ST2 gene products in the human leukemic cell line UT-7 using monoclonal antihuman ST2 antibodies" HYBRIDOMA, NEW YORK, USA, vol. 14, no. 5, October 1995, pages 419-27, XP002101452 cited in the application see page 420, right-hand column, line 35 - page 421, left-hand column, line 1-15 see page 424, line 1-15 see page 426-427 --- -/--	1,2,5,7, 19,22,23

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 April 1999	21/05/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Le Flao, K

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/GB 98/03913

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	KUMAR S. ET AL.: "ST2/T1 protein functionally binds to two secreted proteins from Balb/c 3T3 and HUVECs but does not bind IL-1." THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 46, 17 November 1995, pages 27905-13, XP000604708 see page 27910, left-hand column, line 4-20 ---	1,7
X	US 5 576 191 A (IMMUNEX CORPORATION) 19 November 1996 see column 2, line 20-38 ---	23
P,X	XU D. ET AL.: "Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells." J. EXP. MED., vol. 187, no. 5, 2 March 1998, pages 787-794, XP002101601 see the whole document ---	1-3,7-9, 11-23
P,X	LÖHNING M. ET AL.: "T1/ST2 is preferentially expressed on murine Th2 cells, independent of IL-4, IL-5, and IL-10, and important for Th2 effector function." PROC. NATL. ACAD. SCI. USA, vol. 95, no. 12, June 1998, pages 6930-35, XP002101454 see the whole document -----	1,5,7-9, 12-14, 19-23

INTERNATIONAL SEARCH REPORT

Ir...national application No.
PCT/GB 98/03913

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 10 and 11 (both partially, as far as an in vivo method is concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Interr ıal Application No
PCT/GB 98/03913

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5576191 A	19-11-1996	NONE	